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Molecular Biological Analysis of the Effects of Ginsenoside-Rb_2 on Albumin mRNA in Streptozotocin-induced Diabetic Rats

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Abstract

The mechanisms of the mRNA synthesis-promoting action of ginsenoside-Rb_2, were investigated at the gene level.

Rot analysis suggested that the previously reported increase in RNA polymerase activity as a result of administration of ginsenoside-Rb_2 might be because of its effect on a specific gene. In this regard, albumin mRNA, which is expressed specifically in the liver, was assayed by northern blot hybridization using albumin cDNA in normal rats, diabetic control rats and diabetic rats given ginsenoside-Rb_2. When the level of albumin mRNA in normal rats was set at 100, the level was reduced markedly to 32 in diabetic control rats. In contrast, in diabetic rats given ginsenoside-Rb_2 the level was 0·54, significantly higher (69%) than that in diabetic rats given no ginsenoside-Rb_2. In addition, poly(A)$^+$RNA was purified from total RNA and subjected to hybridization, and poly(A)$^+$RNA bands with different charges were measured by densitometry. The results of the measurement revealed changes dependent on the charge, and this was confirmed by autoradiography.

We found no significant difference in the transcription activity of albumin mRNA, however, it showed only a tendency to increase. This suggests that ginsenoside-Rb_2 has some effect on post-transcriptional regulation of the stability of mRNA itself. The results of Rot analysis suggest that ginsenoside-Rb_2 affects a specific gene alone.

Dysbolism induced by diabetes involves not only the metabolism of carbohydrates and lipids but also that of proteins. In patients with diabetes, therefore, even when food is ingested the metabolic pattern of the body falls into the hunger type because of insulin deficiency. The hunger-type regulatory mechanism, although trying to maintain homeostasis, generates a vicious circle, resulting in an intractable condition accompanied by various complications (Bondy 1971).

We have previously demonstrated that ginsenoside-Rb_2—a saponin contained in ginseng, which has been used since ancient times for its tonic, aphrodisiac, hematopoietic, heat-retaining and refreshing actions—can normalize such dysbolism to some extent in streptozotocin-induced diabetic rats. In particular, because ginsenoside-Rb_2 has been found to stimulate protein and RNA synthesis in the liver and to improve nitrogen balance, it has been suggested that the compound would promote somatic protein synthesis (Yokozawa & Oura 1990; Yokozawa et al 1985a, b, 1987a, b, 1989, 1993a). In addition, because ginsenoside-Rb_2 increases nuclear RNA polymerase activity and the quantity of ATP, the compound is speculated to have some influence on mRNA (Yokozawa et al 1991, 1993b).

Accordingly, the effect of ginsenoside-Rb_2 on mRNA (albumin mRNA) in rats with streptozotocin-induced diabetes has been analysed using a molecular biological procedure.

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Materials and Methods

Saponin

Ginsenoside-Rb_2 was isolated and purified from a root extract of *Panax ginseng* C. A. Meyer produced in Kumsan, Korea; a voucher specimen of the plant was deposited in the Herbarium of Toyama Medical and Pharmaceutical University, Toyama, Japan. The structure of ginsenoside-Rb_2 has been established previously by Sanada et al (1974) as (20*S*)-protopanaxadiol-3-[*O*-β-D-glucopyranosyl(1 → 2)-β-D-glucopyranoside]-20-[*O*-α-L-arabinopyranosyl(1 → 6)-β-D-glucopyranoside] (Fig. 1).

Animals and treatment

Diabetes was induced in male Wistar rats (200 g) by intraperitoneal administration of streptozotocin (50 mg kg^{-1}) dissolved in 10 mM citrate buffer (pH 4·5) (Junod et al 1967). Two weeks after injection, blood glucose was determined, and rats with a glucose level of 390–420 mg dL^{-1} were used as diabetic models. Ginsenoside-Rb_2 (50 mg kg^{-1} per day)

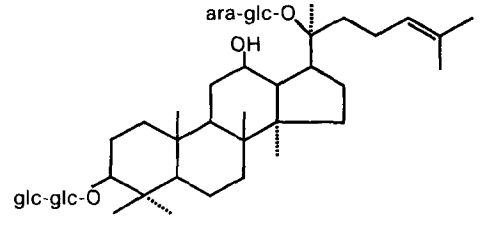

Fig. 1. Structural formula of ginsenoside-Rb_2.

dissolved in saline was administered intraperitoneally to rats for 1, 3 or 6 days, while control rats were given an equal volume of saline. Four hours after the last treatment, the rats were decapitated, and the liver was removed quickly and placed in liquid nitrogen.

Preparation of cDNA

The recombinant plasmid, pTZ19U, containing a 1110-nucleotide sequence specific for rat albumin mRNA, was kindly provided by Dr H. Esumi (Biochemistry Division, National Cancer Research Center Institute, Tokyo, Japan). Cloned rat albumin cDNA was purified by the method of Dretzen et al (1981).

RNA preparation and northern blot analysis

Total RNA was isolated from rat liver as described by Chirgwin et al (1979). Poly(A)$^+$RNA was purified using oligo-d(T) cellulose chromatography. Total RNA (20 mg) and various amounts of poly(A)$^+$RNA were subjected to 1·5% formaldehyde–agarose gel electrophoresis and transferred to Gene Screen Plus R (Du Pont). The hybridization was performed as suggested by the manufacturer. The blot was probed with the EcoRI fragment of pTZ19U-RSA695, which was labelled by the nick-translation method as a standard protocol (Sambrook et al 1989). The filter was exposed to X-ray film at −80°C. For quantitation of individual mRNAs, films were exposed for periods during which band intensity was linear with respect to time, and the quantitation of total RNA and relative intensities of hybridization signals were determined at 480 nm with a Hoefer GS 300 spectrophotometer (USA).

Rot analysis

Poly(A)$^+$RNA was prepared from rat liver as described above. Rot analysis was performed as described by Kameji et al (1977). Single-stranded cDNA was primed with oligo-d(T)$_{18}$ primer as a probe. The reverse transcription mixture included α-^{32}P-dCTP and M-MLV reverse transcriptase, and incubation was performed at 37°C for 1 h. Template poly(A)$^+$RNA in the labelled cDNA mixture was hydrolysed by addition of NaOH. The product of cDNA synthesis was then purified by Sephadex G-50 chromatography. The hybridization reaction was performed using a reaction mixture of 0·6 M NaCl, 13 mM Tris-HCl (pH 7·4), 0·13 mM EDTA, 50% formamide and 1500 counts min^{-1} of labelled cDNA and heated at 95°C for 10 min to denature the poly(A)$^+$RNA and labelled cDNA. The reaction mixture was then incubated at 41°C for various times to determine the kinetics of the formation of cDNA-poly (A)$^+$RNA heterodimer. The duplex in the reaction mixture at different times was digested at 37°C with 1000 units S1 nuclease and adsorbed on a GF/C filter (Whatman). The filter was then washed twice with washing buffer (5% trichloroacetic acid, 30 mM sodium pyrophosphate) and the radioactivity measured with a scintillation counter.

Nuclear transcription assay

Nuclei were isolated from rat liver as described by Gorski et al (1986). Nuclear transcription assay was performed as described by Marzluff (1978), with small modifications. In brief, purified nuclei were suspended in reaction buffer (10 mM Tris-HCl (pH 8·0), 0·5 mM $MgCl_2$, 300 mM KCl, 0·5 mM each ATP, GTP, CTP, 100 μCi α-^{32}P-UTP) and incubated at 37°C for 30 min. The reactions were stopped using termination buffer (20 mM Tris-HCl (pH 7·5), 2% SDS, 10 mM EDTA, 200 μg mL^{-1} proteinase K) and synthesized RNA as a probe was extracted with phenol and chloroform. The filter, to which linearized pTZ19U-RSA695 was blotted, was hybridized with 8·51 × 10^6 counts min^{-1} of labelled RNA in 6 × SSC, 5 × Denhart's solution, 0·5% SDS, 50% formamide and 250 μg mL^{-1} heat-denatured salmon sperm DNA at 42°C for 10 h following a washing procedure with 0·1 × SSC and 0·5% SDS. A similar protocol was carried out using the filter, on to which the pTZ19U vector was blotted as a control. The filters were exposed to X-ray film at −80°C with an intensifying screen. Quantitation of specific albumin mRNA was performed by measuring the radioactivity on the filter with a scintillation counter.

Statistics

Statistical analysis of the data was performed using the Student's *t*-test.

Results

Analysis of quantitative and qualitative variations in the poly(A)$^+$RNA group

Poly(A)$^+$RNA was purified from total RNA. The poly(A)$^+$RNA recovery rate was calculated to be 0·76% for diabetic control rats and 1·06% for diabetic rats given ginsenoside-Rb$_2$. cDNA was then synthesized from poly(A)$^+$RNA of diabetic control rats, and hybridized with mRNA from diabetic control rats or diabetic rats given ginsenoside-Rb$_2$ for application to Rot analysis (kinetic analysis of cDNA–mRNA hybridization). Since the S1 nuclease resistance just before the start of the reaction was about 7%, with a radioactivity of 102 counts min^{-1} compared with the total 1500 counts min^{-1}, the hybridization rate (%) was calculated by subtracting this background value. When the reaction was allowed to proceed to the point where the Rot (mol s L^{-1}) was 1500, the rate of hybridization (cDNA–mRNA [D–D]) in diabetic control rats was 87·7%, whereas the corresponding rate was 70·5% in ginsenoside-Rb$_2$-treated diabetic rats, a difference of 17·3%. The hybridization rate between cDNA and its matrix, mRNA

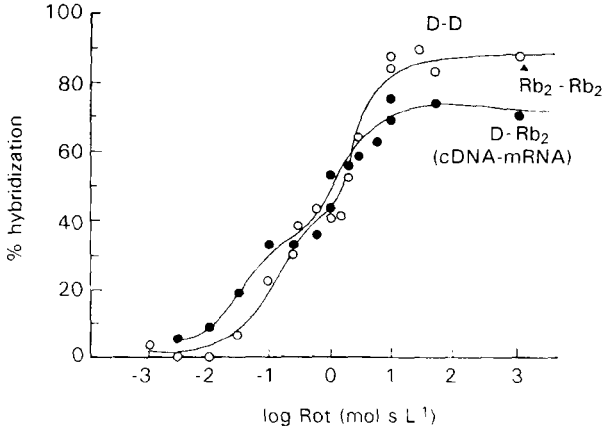

Fig. 2. Hybridization between cDNA and mRNA. D, diabetic control rats; Rb$_2$, diabetic rats given ginsenoside-Rb$_2$.

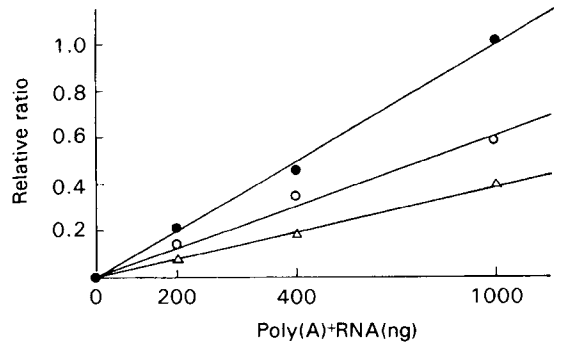

Fig. 3. Albumin mRNA levels in rats receiving intraperitoneal ginsenoside-Rb_2 (50 mg kg^{-1} per day) in saline for 6 days. ● Normal rats, ○ diabetic rats given ginsenoside-Rb_2, △ diabetic control rats.

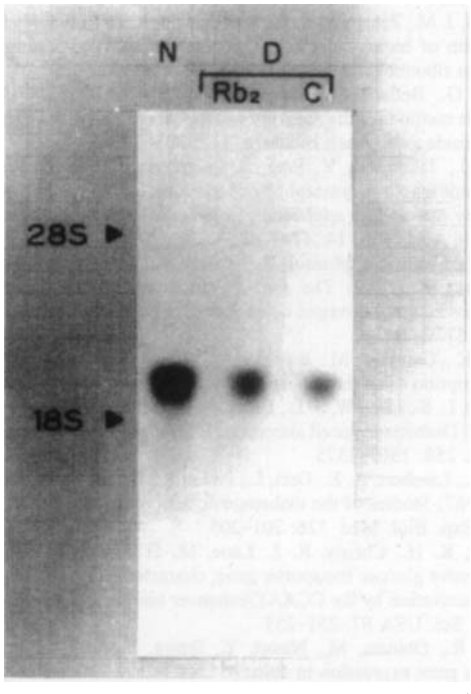

Fig. 4. Typical case of northern blot hybridization of poly(A)$^+$RNA. N, normal rats; D-Rb_2, diabetic rats given ginsenoside-Rb_2; D-C, diabetic control rats. Ginsenoside-Rb_2 (50 mg kg^{-1} per day) dissolved in saline was administered intraperitoneally for 6 days.

(ginsenoside-Rb_2–ginsenoside-Rb_2), in the liver in rats given ginsenoside-Rb_2 was 86·6% at Rot (mol s L^{-1}) = 1500 (Fig. 2).

Albumin mRNA level

If the albumin mRNA level obtained from the total RNA of normal rats was taken as 1·0, the corresponding level in diabetic control rats was 0·32 ± 0·04, showing a distinct decrease of 68% ($P < 0·001$). In contrast, the level in diabetic rats given ginsenoside-Rb_2 was 0·54 ± 0·04, significantly higher (69% $P < 0·01$) than that in the diabetic rats given no ginsenoside-Rb_2. Poly(A)$^+$RNA was purified from total RNA, and the bands of different charge were measured by densitometry. It was found that the albumin mRNA level was 58% higher in ginsenoside-Rb_2-treated diabetic rats than in diabetic control rats (Fig. 3).

Table 1. Nuclear transcription of the albumin gene.

Group	Input (counts $min^{-1} \times 10^6$)	Filter bound (counts min^{-1})
Normal	8·51	1482 ± 103
Diabetic		
Control	8·51	634 ± 75*
Rb_2 (1 day)	8·51	605 ± 62*
Rb_2 (3 days)	8·51	738 ± 76*
Rb_2 (6 days)	8·51	700 ± 32*

Values are mean ± s.e. of six rats. *$P < 0·001$ compared with normal rats.

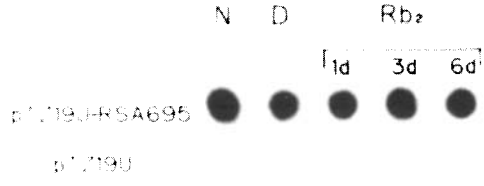

Fig. 5. Nuclear transcription assay of albumin gene. N, normal rats; D, diabetic control rats; Rb_2, diabetic rats given ginsenoside-Rb_2. Ginsenoside-Rb_2 (50 mg kg^{-1} per day) dissolved in saline was administered intraperitoneally for 1, 3 or 6 days.

Fig. 4 shows an autoradiogram of albumin mRNA obtained by the northern hybridization technique of poly(A)$^+$RNA.

Transcription activity of albumin mRNA

The quantity of RNA synthesized in nuclei was $8·51 \times 10^6$ counts min^{-1}/nuclei from 1 g tissue. The radioactivity bound to albumin cDNA was 1482 counts min^{-1} in normal rats. With actinomycin D (100 mg mL^{-1})-treated nuclei, the transcription activity was suppressed by about 90% to 145 counts min^{-1}. The radioactivity obtained with the pTZ19U vector as a probe was at a negligibly low level of 35 counts min^{-1}. Thus, the aforementioned radioactivity in normal rats was thought to reflect the transcription activity of albumin mRNA. On the other hand, the transcription activity in diabetic control rats was reduced by 57% to 634 counts min^{-1} in comparison with normal rats (Table 1), whereas for diabetic rats given ginsenoside-Rb_2 for 3 or 6 days the level was slightly higher than that in the diabetic rats given no ginsenoside-Rb_2. Fig. 5 shows the results of autoradiography after hybridization of labelled RNA and cDNA.

Discussion

On the basis that protein synthesis depends on RNA, a reduction in total RNA has been speculated as a mechanism responsible for reduced protein synthesis in diabetic rats. Since most RNA is rRNA, reduced RNA synthesis leads to a decrease in rRNA, resulting in diminished ribosome formation and reduced ability to synthesize protein. Another possible mechanism is quantitative and qualitative changes in mRNA under the conditions occurring in diabetes (Peavy et al 1978; Jefferson et al 1983).

We have previously reported that ginsenoside-Rb_2 increased the activity of RNA polymerase I and II among various types of RNA polymerase suppressed under diabetic conditions, and

inferred that ginsenoside-Rb_2 would restore the protein synthesis suppressed as a result of the effects on both rRNA and mRNA (Yokozawa et al 1993b). In the present study aimed at examining the transcription pattern of mRNA, one of the two factors involved in ginsenoside-Rb_2 action, we performed Rot analysis and found that the previously reported increase in RNA polymerase activity as a result of administration of ginsenoside-Rb_2 might be the result of an effect on a specific gene, rather than on transcription as a whole. Then, with further attention focused on albumin mRNA, which appears specifically in the liver, the reduced level of albumin mRNA under diabetic conditions was found to increase significantly after ginsenoside-Rb_2 administration. Since total RNA and rRNA decrease in diabetes, poly(A)$^+$RNA was purified to exclude the effect of these decreases, and changes in albumin mRNA were observed closely to investigate its action. Thus, poly(A)$^+$RNA purified from total RNA was subjected to northern blot hybridization using various charging levels, and albumin mRNA was measured by densitometry. The albumin mRNA level revealed changes dependent on the charge. There was, however, no clear-cut change in the transcription activity of albumin mRNA; it showed only a tendency to increase. This finding suggests that ginsenoside-Rb_2 has some effect on post-transcriptional regulation of the stability of mRNA itself due to prolongation of the intracellular half-life of albumin mRNA, and the decomposition of mRNA by RNase.

Among these three factors, an increase in the stability of albumin mRNA is less probable. Although the rate of recovery of poly(A)$^+$RNA from an oligo(dT)-cellulose column was increased in ginsenoside-Rb_2-treated diabetic rats, polymorphism of mRNA as a result of differences in signal recognition is unlikely to be involved because there is only one poly A signal in the N exon of the rat albumin gene, as demonstrated by Esumi et al (1982); in addition, there was no AUUUA sequence, which is known to contribute to the stability of mRNA of various cytokines (Shaw & Kamen 1986). On the other hand, the 3′ non-translational sequence of mRNA has a secondary structure, forming a peculiar loop, to which protein factors bind to protect it against RNase (Mullner et al 1989). It seems reasonable that ginsenoside-Rb_2 exerts some action on this structure.

In recent years, detailed studies of the control mechanisms for expression of various genes have ruled out the idea that there are transcription factors specific to individual genes. It is now believed that a combination of several transcription factors determines the expression of a particular gene. With regard to the albumin gene, which was examined in the present study, Frain et al (1989) and Landschulz et al (1988) pointed out that its promoter activity is controlled by a combination of HNF-1 present in the liver and kidneys, C/EBP specific to fatty tissue and NF-1 having no tissue specificity. Akira (1990) and Kaestner et al (1990) have, on the other hand, reported that C/EBP is involved not only in the expression of the albumin gene but also in the transcription of the stearoyl-CoA desaturase I and insulin-reactive glucose transporter (GLUT4) genes, which are expressed specifically in the liver. In the present study, the results of Rot analysis suggested that ginsenoside-Rb_2 affects a specific gene alone.

Considering the control mechanisms responsible for the expression of the albumin gene, it remains unclear, however, whether the gene induced by ginsenoside-Rb_2 is actually the albumin gene alone, whether there are some other such genes, and what transcription factor takes charge of the expression and induction of the gene(s). These issues require further investigation, particularly through cloning of the genes induced by ginsenoside-Rb_2.

Acknowledgement

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